A RESEARCH PROTOCOL TO DETERMINE: ORIGINAL

The Efficacy of Oxytetracycline Medicated Feed to Control Mortality Caused by Flavobacteriosis in a variety of Fish Species.

Protocol OTF - 98 - EFF

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Testing Facility: Federal, State and Private Facilities throughout the United States

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1. INTRODUCTION:

1.1 Objective:

1. To evaluate the efficacy of oxytetracycline medicated feed fed at a rate of 2.5 - 10.0 grams of active drug per 100 pounds of fish per day for up to 14 days to control mortality caused by Flavobacteriosis in a variety of fish species.

2. H_o :Mortality caused by flavobacteriosis is equal between fish groups treated with oxytetracycline medicated feed fed at a rate of 2.5 - 10.0 grams of active drug per 100 pounds of fish per day for up to 14 days, and fish groups than receiving unmedicated feed.

3. H_a : Mortality associated with flavobacteriosis will be lower among fish groups treated with oxytetracycline medicated feed fed at a rate of 2.5 - 10.0 grams of active drug per 100 pounds of fish per day for up to 14 days than among fish receiving unmedicated feed.

4. Investigator, study monitor and hatchery personnel involved in the conduct of the study will be thoroughly familiar with both the Implementation Guide for Oxytetracycline - Feed Additive INAD #9006 by the USFWS, the Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Oxytetracycline - Feed Additive INAD #9006 (see Attachment I); the Study Protocol for the Efficacy of Oxytetracycline Medicated Feed to Control Mortality Associated with Flavobacteriosis in a variety of Fish Species - Protocol OTF-98-EFF; and the Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (FDA document dated Oct. 1992 or later - see Attachment II).

1.2 Background and Justification:

In addition to the use patterns listed on the current label, oxytetracycline historically has been the drug of choice when diagnostic evidence shows salmonids to have enteric redmouth caused by *Yersina ruckeri*, flavobacteriosis caused by *Flavobacter columnare, Flavobacter psychrophilus*, or closely related yellow pigmented gliding bacteria as described in the U. S. Food and Drug Administration (FDA) Public Master File #5456; or, vibriosis caused by *Vibrio anguillarum, Vibrio ordalli* or other closely related bacteria. Oxytetracycline has also been useful for the control of enteric redmouth septicemia of catfish caused by *Edwardsiella ictaluri* and for bacterial hemorrhagic septicemia caused by

Aeromonas (liquefaciens) hydrophilia and other closely related bacteria, pseudomonas diseases caused by *Pseudomonas sp.*, or flavobacteriosis in sturgeon, paddlefish, temperate bass, catfish, goldfish, and other fish species including some fish species listed as threatened and endangered under the Endangered Species Act.

Integrated fish health management practices usually prevent the occurrence of these diseases. However, adverse environmental conditions, physiological changes related to smoltification or the onset of spawning, uncontrollable water supplies, and unforeseen factors can lead to severe disease outbreaks requiring prompt treatment to prevent significant losses in excess of 50% of fish valuable of natural resource stewardship. Such treatment also reduces the discharge of infectious agents into the natural environment thereby reducing the spread of disease.

Treatment strategies for the use of oxytetracycline in fish shall be designed to meet the needs of each species or lot, the size and numbers of fish to be treated, the layout of the facility, and environmental conditions. In all cases the objective shall be to minimize the impacts of disease on fish health, fish quality and survival and to fully meet fishery management objectives. Because there are many factors that can affect the success or failure of oxytetracycline therapy, pivotal efficacy data is needed that will determine the best ways to use the drug.

The purpose of this Study Protocol is to obtain pivotal clinical field efficacy data demonstrating the value of oxytetracycline therapy in a variety of environmental conditions, at a wide range of temperatures, and in a variety of cultured fish species. This data is critical in extending the labeled use pattern to cover the control of additional diseases in additional fish species under a broader spectrum of environmental conditions. Many of the studies on fish species or fish diseases not now covered by the existing drug label will be conducted with oxytetracycline use patterns identical to those already approved for use on salmonids and catfish. By doing so, studies carried out as described in this protocol will present fewer risks to human food safety, the environment, or to target animals.

To date, there exists limited data evaluating treatment concentrations and durations that exceed approved label use. This Protocol establishes maximum treatment concentrations to 10.0 grams active drug/100 lbs fish/day, and treatment durations not to exceed 14 days. Initial attempts at pivotal studies will focus on treatment concentrations not to exceed those currently approved by FDA. However, if opportunities arise to

evaluate higher treatment concentrations/longer treatment durations, they will be pursued with full knowledge that at this time, supporting residue depletion, target animal safety, etc. data may not exist in their entirety.

The objective of these field based clinical efficacy trials is to evaluate the efficacy of therapeutic oxytetracycline medicated feed treatment regimes to control mortality is a variety of fish species caused by flavobacteriosis at a number of study sites. Fish size in most studies will range from 1 - 4 inches in length. Fish in this size range are typically reared during a time of year in which the prevalence of pathogens associated with flavobacteriosis is highest, and disease outbreak is most likely to occur.

Efficacy studies will be conducted at facilities after the following have been considered: 1) study sites have historical, predictable, recurring outbreaks of fish diseases of interest; 2) each has space at the hatchery to dedicate to conducting studies; 3) each have additional resources available to conduct studies (i.e. - test units, test animals, staff to monitor study and collect data); 4) and each has demonstrated commitment to adhere to Protocols and Guidelines. Studies will be conducted under compassionate Investigational New Animal Drug exemption #9006 and Pivotal Protocol OTF - 98 - EFF and are intended to provide the FDA/Center for Veterinary Medicine (CVM) with pivotal clinical field efficacy data.

2. INVESTIGATIONAL DRUG AND CONTROL:

2.1 Test Substances:

2.1.1 Trade name:
Terramycin (oxytetracycline hydrochloride)

2.1.2 Chemical name - active component(s):
oxytetracycline (CAS No. 79572) quaternary salt

2.1.3 Molecular formula
4-dimethylamino-1, 4, 4a, 5, 5a, 6, 11, 12a-octahydro-3, 5, 6, 10, 12, 12a-hexahydroxy-6-methyl-1, 11-dioxo-2-naphthacene-carboxamide hydrochloride

2.1.4 Appearance and odor:
Uniform tan meal with a cereal odor

Color and crystalline form:

Yellow platelets

2.1.5 Active/inactive ingredients

oxytetracycline (CAS No. 79572) quaternary salt

2.1.6 Dosage Form

Oxytetracycline medicated feed for use in treating flavobacteriosis in fish is insoluble in water and is added to commercial or manufactured fish feeds. The drug contains oxytetracycline quaternary salt in the following concentrations: 100 grams active drug per pound of Terramycin 100 or 100D.

2.1.7 Dose(s) to be tested

2.5 - 10.0 grams oxytetracycline per 100 lbs of fish per day.

2.1.8 Manufacturing site

Pfizer, Inc.
1107 South 291 Highway
Lee's Summit, MO 64081

2.1.9 Lot Number

Studies will be conducted at numerous sites, and Terramycin 100 and 100D will be purchased separately by each facility. Therefore, Terramycin lot numbers will be listed on Form 3 of the Pivotal Protocol and in the methods section of the final report submitted FDA/CVM following each field based clinical study.

2.1.10 Packaging

Oxytetracycline medicated feed will be stored in its original packaging. Medicated feed usually arrives on station in large plastic bags vacuum packed with ethoxyquin gas. Unopened bags of dry feed will be stored in a cool dry place, and moist feed will be stored frozen. If feed is top dressed with Terramycin, bulk chemical will be weighed out, dissolved in hot water, mixed with a gelatin and sprayed on feed to achieve the desired number of grams active compound per pound of feed. Feed and Terramycin will be mixed in a large (cement-type) mixer, poured back into original feed bags, sealed, labeled and stored according to feed type.

2.1.11 Storage conditions

Treated feed will be stored at temperatures and for periods of time not to exceed limits set by the feed manufacturer. Treated feed

should be ordered only as needed and not stored for possible future use.

2.1.12 Drug storage during study.

Oxytetracycline medicated feed will be stored in its original container supplied by the feed manufacturer with the appropriate investigational label attached. Opened containers will be stored in a cool, dry location away from direct sunlight, or if dictated by feed type, in a storage freezer.

2.1.13 Drug handling procedures:

Each Study Monitor and Investigator will be provided with a current copy of the Material Safety Data Sheet (MSDS) for Oxytetracycline (Terramycin; Appendix I). Each person involved with the study and each person who may be present during the use of oxytetracycline medicated feed shall be required to read the MSDS. Safety precautions as outlined in the MSDS will be followed at all times when working with the drug.

The possible hazards associated with handling of oxytetracycline medicated feed should be discussed prior to initiation of the study. Individuals with known allergic reactions to oxytetracycline will not be permitted to handle such feed. For transportation emergencies telephone CHEMTREC at 800/424-9300.

2.1.14 Verification of drug integrity/strength:

Pfizer Inc. will provide limited analytical support to determine if oxytetracycline dosages in formulated feeds meet specifications. Investigators must record treated feed lot numbers on Form 1 Guide for Reporting Investigational New Animal Drug Shipments for Poikilothermal Food Animals, as well as on Form 3 Diagnosis, Treatment, and Mortality Record for Clinical Field Trials using Oxytetracycline. Feed will also be assayed for drug strength by the Upper Midwest Environmental Science Center (UMESC), United States Geological Service, Biological Research Division (see Section 6.5.1.1.4 for address and telephone numbers).

Commercial fish feed manufacturers shall prepare fish feed with dosages of oxytetracycline quaternary salt according to concentrations desired by the Investigator at the study facility. Feed manufacturers shall prepare drug-treated feed by adding Terramycin 100 For Fish or Terramycin 100D premix to feed.

The Investigator may also prepare his/her own drug-treated feed by top dressing feed with Terramycin to achieve desired dosages. If the Investigator chooses this option, they must have a sample of the top dressed feed assayed for oxytetracycline concentrations by a certified, analytical test laboratory (e.g., UMESC). Results of drug-treated feed assays will be included in the final reported to FDA.

2.1.15 Investigational labeling:

Copies of the labels to be attached to each container of oxytetracycline medicated feed will be provided to all facilities participating in pivotal clinical field efficacy trials. It will be the responsibility of the Investigator to ensure proper labeling of all containers of oxytetracycline medicated feed.

2.1.16 Accountability:

Each Investigator will notify FDA prior to any shipment of oxytetracycline medicated feed for use under this INAD. Immediately upon placing an order with the approved supplier, the Investigator will complete Form 1, "Guide for Reporting Investigational New Animal Drug Shipments for Poikilothermic Food Animals" and send it to the Study Monitor. The Study Monitor will then send the original plus two copies to the FDA. Both the Investigator and Study Monitor are required to sign Form 1. The Study Monitor will send a single copy of Form 1 to the Study Director at the Bozeman National INAD Office (NIO). The Investigator will keep one copy of the completed Form 1 for the facilities INAD file. Arrangements should be made between Investigators and Monitors to insure completed Form 1's are received by the FDA within 7 days of the date an order was placed.

Investigators are also responsible for maintaining an accurate inventory of oxytetracycline medicated feed on hand. A chemical Use Log (Form 2) will be supplied by each Investigator. Each time oxytetracycline medicated feed is used, it must be reported by the Investigator on Form 2.

At the conclusion of the study, all remaining oxytetracycline medicated feed will remain on site for further use, or be discarded in the local landfill. The properly recorded Chemical Use Log (Form 2) will be sent to the Study Monitor.

2.1.17 Material Safety Data Sheet (MSDS)

A complete MSDS is included in Appendix I.

2.2 Controls:

Controls will be fish that receive non-medicated feed. Feed fed to controls will be administered in a manner identical to the treated fish with respect to feed amounts and method of feeding.

3. STUDY SCHEDULES:

3.1 Proposed date(s) of initiation:

Proposed dates of initiation will be study site specific. Studies will be conducted at several U. S. Fish and Wildlife Service (Service) facilities, as well as State Hatcheries and private facilities yet to be determined. These locations have a history of predictable, recurring outbreaks of flavobacteriosis (coldwater disease or columnaris). Studies will be initiated when there is a disease outbreak and the Study Director and Investigator is available to initiate oxytetracycline medicated feed treatment. There is a very good probability that more than one site will experience a disease outbreak at approximately the same time, and the Study Director will be unable to be on-site to conduct the study. In the event this should occur, the Investigator on station will initiate the study with telephone support from the Study Director. Dates for all studies will be summarized in the final report.

3.2 Schedule of events:

3.2.1 Transfer of test animals:

Fish will be transferred to tanks/raceways shortly after the swim-up stage (termed "ponded") or when a raceway or tank of fish begin to show disease symptoms (described in Section 9.2 Inclusion criteria). Fish will be transferred into test units no less than 24 h prior to initiation of treatment. All information pertaining to transfer of fish to test units, including dates of transfer and study initiation, will be described in the methods section of the final report.

3.2.2 Initiation of treatment:

Treatment will begin no sooner than 24 hours post-transfer of fish to test units, or for fish ponded after swim-up, when the disease/pathogen has been confirmed.

3.2.3 Treatment period duration:

The treatment period will begin the day treatment is initiated (day 1) and will last no more than 14 days.

3.2.4 Post-treatment period duration:
Duration of the post-treatment period will be at least 14 d, but may be as long as 30 days.

3.2.5 Data analysis and report writing:
Data collection, analysis and preparation of final report will require a maximum of 12 months post-study termination to complete. Within 12 months of completion of post-treatment period, a final report will be submitted to the Food and Drug Administration/Center for Veterinary Medicine.

3.3 Proposed date(s) of completion:
The data collection portion of most individual studies will be completed within 45 d of the initiation of treatment. Final date of completion of individual studies will be approximately 12 months after completion of the study. The proposed date for completion of all studies will be December 2003.

4. STUDY DESIGN:

4.1 Treatment groups:
Studies will typically consist of a single treated group and a single non-treated (control) group. However, objectives of some studies will be to evaluate treatment efficacy of 2 or more treatment regimes. All fish for a particular study will consist of fish from the same fish lot. Study fish will be reared in either a single common rearing unit or in multiple units under the same environmental and culture conditions. Treated groups will be treated at 2.5 - 10.0 grams active drug per 100 lbs fish per day. Control groups will receive no chemical treatment, but will receive non-medicated feed at the same feed rate as the treated fish. There will be at least three replicates per treatment group.

4.2 Experimental design:
The experimental design used will be completely randomized.

4.3 Blocking factor(s):
It is anticipated that no blocking factor(s) will be used in the study design. All rearing units will be plumbed with the same water supply in a confined area in a hatchery building or bank of raceways. All units will be exposed to the same ambient temperature and photoperiod, and fed the same diet.

Water chemistry conditions, such as temperature, dissolved oxygen, pH, and hardness should be consistent among all rearing units. All rearing units will experience similar foot traffic and light conditions. However, if blocking is deemed appropriate, a description will be included in the final report.

4.4 Randomization procedures:

4.4.1 Allocation of animals to test units:

Animals will be randomly placed in one of six test units in such a manner as to minimize bias (presuming there is only a single treated group). The test units will be numbered 1 through 6. Fish should be moved to test units in stages to minimize bias. At each stage, a given test unit will receive no more than approximately 25 - 50% of the total number of fish to be ultimately transferred to the test unit. A random number table or blindly drawing one of six cards from a bucket (3 labeled treated, 3 labeled control) without card replacement will be used to determine which test unit will receive the first group of fish, which will receive the second, and so on until fish have been transferred into all test unit. Following this procedure, each test unit will receive a second, third and final group of fish following the same order as described above.

4.4.2 Allocation of treatment groups to experimental units:

After all fish have been transferred to test units, units will be randomly assigned as either Treated or Control using the same procedure as described in Section 4.4.1.

4.5 Configuration of experimental units:

Configuration will be different at each facility. Each test unit will be configured and plumbed in the same manner, each will receive water from the same source and hold equal numbers of test fish. The configuration of experimental test units will be described in detail in the methods section of the final report.

5. STUDY PROCEDURES:

5.1 Test Animals:

5.1.1 Description:

5.1.1.1 Age/Size:

Fish used in the studies will be fry/fingerling, less than 6 months old and between 1 - 4 inches in length (see Table 5.1.2). Fish species, age and size will be described in detail in the final report.

5.1.1.2 Sex:
Sex of fish used during the study will not be determined. It is assumed that approximately 50% of the test fish will be males and 50% of the test fish will be females. It is also assumed that sex will not be a factor in treatment efficacy as fish will not be sexually mature.

5.1.1.3 Breed/Class:
Fish species will be confirmed by the Hatchery Manager.

5.1.1.4 Initial body weight:
As explained in Section 5.1.1.1, age and size of fish to be used in the studies are not known at this time, and will be described in the final report.

5.1.1.5 Physiological state:
Test fish will be in no particular physiological state. In all cases, test fish will not be sexually mature (hatchery reared fish less than 4 inches in length at the start of the study).

5.1.1.6 History of test animals:
Test fish will most likely be from eggs incubated at the study site. Test fish will typically be reared on site under standard hatchery conditions as described by Piper et al. (1981). A brief description will be included in the final report detailing egg source, egg incubation procedures and site if different than fish test site, management practices and environmental conditions under which test fish were reared prior to study (e.g., type and size of fish, flow and density index values, water turnover rates, rearing temperature, pH, hardness, dissolved oxygen), and any therapeutic chemical treatment administered prior to the study. If such treatment has been deemed necessary, a justification statement for the treatment will be included in the final report.

5.1.2 Number of test animals:
The number of test animals used in each study will be predicated on achieving a flow index and density index that approximates normal production conditions. Use of these indices, which are described below (see Tables 5.1.2.1 & 5.1.2.2), will be used instead of absolute fish

numbers. This will be done to achieve a degree of uniformity among all study sites. It is likely that the various study sites will use various sizes and shapes of rearing units (e.g. 3 - 6' circular tanks or 4 - 16' rectangular troughs) and this will ensure that all studies will be conducted under approximately the same conditions.

5.1.2.1 Flow index:

A flow index (FI) range, in combination with a density index range, will be used to describe the number of test animals used. Note that ranges will differ for different fish species at a given facility. Flow index is the relationship of fish size/weight to the water flow (flow rate) to a rearing unit, calculated by the formula:

$$FI = \frac{\text{(total number of fish)*(mean weight of fish (lbs))}}{\text{(mean length of fish (in.))*(water flow rate (gal./minute))}}$$

As the number of fish decreases in a particular test unit as a result of mortality, the flow index will also decrease. No measures will be taken to maintain flow index as the study progresses to the level set at the start of the study. Although fish will be fed during the course of study, no appreciable growth or weight gain is expected. Water flows will be readjusted periodically throughout the study to maintain constant water flow.

5.1.2.2 Density index:

A density index range, in combination with a flow index range, will be used to describe the number of test animals used. Note that ranges will differ for different fish species at a given facility. Density index is the relationship of fish size/weight to water volume of a rearing unit, calculated by the formula

$$\frac{\text{(total number of fish)*(mean weight of fish (lbs))}}{\text{(mean length of fish (inches))*(size of test unit (in cubic feet))}}$$

As the number of fish decreases in a particular test unit as a result of mortality, the density index will also decrease. No steps will be taken to lower the volume of water in the test unit to maintain the density index as the study progresses at the level set at the start of the study. Although fish will be fed during the course of study, no appreciable growth or weight gain is expected.

5.1.3 Source of animals:

Source of test animals will be recorded in Form 3. At this time the source of test animals is not known but will undoubtedly be different for each participating facility.

5.1.4 Identification method if not client-owned companion animals: Test animals will not possess any artificial or man-made identification. Measures will be taken to ensure test animals from one test unit do not mix with test animals from another test by covering test units with screens or ensuring the distance from one test unit to another is sufficient enough that fish cannot jump from one test unit to another. Because methods may differ among facilities, the method used to prevent fish from moving from one test unit to another will be described in the final report.

5.2 Inclusion criteria:

The entrance criteria for inclusion in the study will include the following: 1) study fish that are diagnosed with flavobacteriosis (coldwater disease or columnaris); 2) fish are known not to have any secondary disease pathogen; 3) the study investigator is on site to initiate transfer of fish and initiate start of treatment; and 3) enough test units and test animals are available to conduct studies in triplicate under production-like conditions. Study Monitors, Investigators and other personnel involved in the study are thoroughly familiar with protocols and guidelines listed in Section 1.1 paragraph 4, and will be committed to following through with the study until completion.

Diagnosis of flavobacteriosis will be confirmed by the following procedure: Use of media such as Shiehs, TSA, TYES or Brain Heart Infusion Agar, as they are best for isolating flavobacters and should be used when coldwater disease (CWD) or columnaris are suspected (Meyers 1997). Note - When CWD is suspected, TYES is the preferred agar of choice. Streak for isolation on agar plates with inoculum from kidney or spleen. Incubate at temperatures and for durations listed below. Another effective technique which may be used for streaking tissue on media for detection of columnaris would be to aseptically remove spleen and streak of mince on agar.

Results:

Flavobacter psychrophilus - Growth of bright yellow colonies at 15 - 18° C with convex center, spread periphery, and fried egg appearance.

Flavobacter columnare - Growth of yellow convoluted centered colonies with rhizoid edges at 20 - 25° C for 2 - 3 days.

In addition, gill (for columnaris) or kidney/spleen imprints can be made to confirm flavobacteriosis by gram stain and by observing cell morphology.

5.2.1 Ability of investigator to fulfill all the requirements of the protocol:

Investigator will be fully capable of ensuring all requirements of the protocol are fulfilled. Study sites were selected, in part, because Investigator(s) have experience using oxytetracycline medicated feed in accordance with the compassionate INAD protocol to treat for flavobacteriosis. Not only will the Investigator be thoroughly familiar with the Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Oxytetracycline - Feed Additive INAD #9006 (see Attachment I), but also the Pivotal Study Protocol for Efficacy of Oxytetracycline Medicated Feed Treatment for the Control of Mortality caused by Flavobacteriosis - Study Protocol OTF - 98 - EFF; and with Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (dated Oct. 1992 or later - see Attachment II).

5.2.2 Level of disease:

Ideally, there should be increased morbidity or mortality rates among fish with disease signs typical of bacterial infections that cannot be treated by other means. Typical disease signs should be detectable in at least a few fish and the causative bacterial agent identified. However, the level of flavobacteriosis must be relatively low or in the early stages of development to obtain control. If the level of the disease is far advanced, chronically infected fish may be produced which may result in complete mortality. Therefore, prompt diagnosis and treatment is imperative. In general, the Investigator will respond with diagnosis and initiation of fish transfer into test units or begin the study if fish are already in test units, when daily mortality rates exceed approximately 0.1% of the total fish in a holding unit.

5.3 Exclusion criteria:

A test unit will be excluded from the study if conditions such as multiple disease outbreaks occur, water flow is interrupted unintentionally for more than a few minutes, a standpipe is left out, or some other similar incident occurs. Fish used in section 5.2 to determine disease uniformity will be evaluated using a standard fish health diagnostic scheme to determine if other infectious pathogens are present (see Appendix V). If it is discovered that mortality among diseased fish may not be attributable to systemic flavobacteria, then steps for exclusion will be taken. If pre-

treatment mortality in any unit is too high, which may possibly jeopardize the integrity of the experimental units with respect to extrapolation to the fish populations sampled, then results from these units may be excluded from statistical analysis. If exclusion of any test unit is deemed necessary, a full explanation will be described in the final report, as will modifications in the data analysis. If an incident occurs during the course of the study which may introduce variability but does not result in exclusion, it will also be described in the final report.

In addition, if differences in mortality between treated and untreated groups is significant, provisions may be made to administer treatment to control fish. However, prior to administering treatment to control fish, approval will be consented by FDA/CVM (Dr. Tom Bell), Study Director and Investigator. Furthermore, if mortality in control test units becomes excessive as perceived by the Hatchery Manager, then these fish may be excluded from the study and treated. A decision to "pull" control fish from the study, possibly jeopardizing the entire study, will most likely be made by the Hatchery Manager, but not without first discussing this with the Study Director and Investigator.

5.4 Acclimation of test animals:

5.4.1 Duration:

Test conditions will be nearly identical to conditions under which fish became diseased. The 24-hour period between transfer of fish to test units and initiation of treatment will be termed the acclimation period. If a longer acclimation period is used, the duration and reasons for instituting the lengthened acclimation period will be described in the final report.

5.4.2 Medication and/or vaccination during acclimation period:

During the acclimation period, no medication and/or vaccinations will be administered to the test animals. In the event medication and/or vaccinations are deemed necessary and administered to test fish, that test unit will be excluded from the study or the study will be terminated.

5.4.3 Baseline data collected prior to initiating study:

Disease level and uniformity, described in Section 5.2.5, will be evaluated prior to initiating study, as will fish length and weight. Mortality data will also be collected from all rearing units prior to initiation of study.

5.5 Blinding of study:

5.5.1 Extent of blinding:

Single blinding technique: Either the Hatchery Manager, Asst. Hatchery Manager or other Investigator will be the only study participant who has prior knowledge regarding experimental unit treatment condition. This same person will also be the only person on-site with access to the codes indicating which test units are treated and which are controls (recorded on Form 8). This person will take responsibility for filling feed containers identified with only their assigned rearing unit. Containers will have no identification indicating whether contents contain medicated or non-medicated feed. All containers will contain the same amount of feed. At this time, others involved in the study may feed test fish but will remain blinded as to which rearing units received medicated feed. The disclosure of who will know treatment codes and who actually feeds test fish will be described in the final report.

If any other blinding technique or deviation to the above described blinding techniques are used, they will be described in detail in the final report.

5.5.2 Blinding method(s) and procedure(s):
A single blinding method will be used (see Section 5.5.1).

5.5.3 List of personnel with access to treatment codes and rationale:
Table 10.2 lists the Investigators and study monitor for each participating facility. Mr. Jim Bowker and Dr. Dave Erdahl will serve as Study Director and Sponsor for all studies. The Investigator, or other study participant, will have access to the treatment codes. The person with access to treatment codes will either be the Hatchery Manager or Asst. Hatchery Manager, or individual assigned this responsibility by the Hatchery Manager/Asst. Manager. This person will possess sufficient scientific background to assist in the study, but will not play a role in the day-to-day activities of the study. One other person with access to treatment codes may be Dr. Erdahl. Dr. Erdahl is the Services National INAD Coordinator, and as Sponsor will have knowledge of each study conducted by the Service, but will not play a role in conducting the study. Treatment condition codes will be recorded on Form 8 by the study participant with access to treatment conditions. When this form has been completed, it will be stored in a secure location not accessible to anyone except the listed person. At study termination, this form will be included with other data/information forms.

5.6 Drug Administration:

5.6.1 Dosing regime:
The dosing regime for all studies will be 2.5 - 10.0 grams active drug/100 lbs fish/day. Fish will be fed daily for no more than 14 days.

5.6.2 Route of administration:
Oral administration (on fish feed)

5.6.3 Investigational withdrawal period:
40 days (see Appendix VI)

5.6.4 Proposed withdrawal period:

5.6.4.1 treatment regime of 2.5 - 3.75 g active drug/100 lbs fish/day for 10 days
Salmonid fish treated in this manner may be released for immediate harvest after a 21d withdrawal period. No withdrawal period should be required for fish that will not be catchable for 21 or more days after release or are illegal for harvest.

5.6.4.1 treatment regime of 3.75 - 10.0 g active drug/100 lbs fish/day for up to 14 days
A 40 day withdrawal period is proposed for fish treated in this manner. No withdrawal period should be required for fish that will not be catchable for 40 or more days after release or are illegal for harvest.

5.7 Removal of subjects from study:

5.7.1 Criteria for removal of subjects from the study:
Only dead or moribund fish will be removed from the test units.

5.7.2 Procedures for removal of subjects from the study:
Dip nets will be used to remove dead animals from test units. Net(s) will be sanitized prior to and after each use.

5.7.3 Fate of removed study animals:
Dead animals removed from the study will be disposed of in a local landfill or incinerated. Fate of live fish removed from the study will be described in the final report.

5.8 Concurrent/concomitant medications/therapies:

There will be no concurrent/concomitant medication/therapy administered during the course of the study. If concurrent/concomitant medication/therapy is deemed necessary and administered, then that test unit will be removed from the study as described in section 5.3.

5.9 General management practices:

5.9.1 Site visits:

Hatchery personnel involved in the study (listed in Section 10.2), and in some cases, Mr. Bowker or other staff from the National INAD Office (NIO) will be on station daily during the course of the study. Staff from the NIO serving as Study Director or co-Investigators in all likelihood will be on station one day prior to the beginning of treatment, and remain on site during most/all of the treatment period. The Study Monitor will be qualified Regional Fish Health Biologist; see Table 10.1 Investigators, study monitor and fish health biologists involved in proposed field based clinical efficacy trials), and will conduct the site visit in person on over the phone according to Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (dated Oct. 1992 or later).

5.9.2 Data Collection:

Data will be collected by only the Study Director, Investigator (other than the investigator with access to treatment codes) and hatchery staff listed in Table 10.2 as other study participants, according to the schedule outline in the protocol. If other individuals are involved in data collection, they will be listed in the final report.

5.9.3 Frequency of monitoring water chemistry parameters, such as water temperature and dissolved oxygen:

Table 5.9.3 lists when and where water chemistry parameters will be measured during the course of the study. To summarize, water temperature and dissolved oxygen will be measured twice daily, at the beginning and end of each workday (e.g.~ 8 a.m. and 3 p.m.). Water hardness and pH will be measured twice during the study, at the beginning and end of the study.

5.9.3.1 Parameters to be measured prior to initiation of study:

Prior to initiation of treatment, the following will be measured and recorded in Form 3: (1) number of rearing units used in the study; (2) number of treated/untreated units; (3) mean fish weight (grams); (4) mean fish length (inches); (5) rearing unit configuration (i.e. - circular, rectangular); (6) rearing unit dimensions; (7) rearing unit

size (ft^3); (8) number of fish/test unit; and (9) water flow (gallons/minute). Flow index and density index values will be calculated based on the above measured parameters.

5.9.3.2 What, when and where parameters will be measured during study:

Table 5.9.3 describes when and where treatment parameters will be measured during the course of the study. Twice daily indicates at the beginning and end of the work day; daily indicates at the beginning of the work day; twice during study indicates at the beginning and end of the study. Dissolved oxygen will be recorded on Form 5, water temperature will be recorded on Form 6. Water pH and hardness will be recorded on Form 7. Space will be provided on Form 7 to record actual mLs of titrant used to change sample color and show calculations to determine mg/L $CaCO_s$.

Table 5.9.3A. Parameters/schedule/location for taking water quality measurements.

Parameter	Schedule	Location
Dissolved Oxygen	Daily[1]	Mid-Depth - tail-end
Water Temperature	Daily[1]	Mid-Depth - any location
Water Flow	Set once, inspected visually daily	at test unit source
pH	Twice during Study[2]	Surface - any location
Hardness (as $CaCO_3$)	Twice during Study	Surface - any location

1. at ~ 8 am
2. at the beginning and midway through the study

5.9.3.3 Procedures and equipment for assessing treatment parameters:

Tables 5.9.3.3 lists the treatment parameters to be measured, the equipment that will be used to measure these parameters, and the cited reference material used to conduct the measurements. All cited reference material for measuring treatment parameters are included in Appendix VII. Water flows will be set at a predetermined rate by calibrating flow from water-pipes plumbed to each tank. Flow will be measured by collecting water in a suitable container for 15 - 60 seconds and extrapolating total volume delivered over 60 seconds.

Table 5.9.3.3. Equipment and Reference Material for Measurement of Treatment Parameters.

Parameter	Equipment	Reference
Dissolved Oxygen	D.O. Meter (YSI model 55) or other meter (to be described in the final report	Owners manual
Water Temperature	D.O. Meter (YSI model 55) or other meter (to be described in the final report	Owners manual
Water Flow	Stop watch and container marked every ½ gallon	n/a
pH	pH Meter HACH Co. EC 10 or other meter (to be described in the final report	Owners Manual
Total Hardness (as $CaCO_3$)	Hach kit reagents	See Appendix VII

5.9.3.4 Calculations for derived data:
Calculations are described in the reference material (see Appendix VII for total hardness).

5.9.4 Frequency of feeding:
Test animals will be fed daily during the entire study. Feed brands and size, amounts and feeding frequency will be specific for the participating facility and fish species used as the test animal. At this time, these parameters are not known. Feeding frequency will be the same as that received by other production fish reared on site. In general, trout and salmon will be fed 2 - 4 times daily by hand. Because it is difficult to firmly establish feed brand and size, amount of feed and daily feeding frequency at this time, they will be described in detail in the methods section of the final report. Whatever the feed brand, size, amount and frequency used at a particular facility, fish in all test units, whether treated or untreated, will be fed the same feed in the same amounts in the same manner.

5.9.5 Frequency of monitoring and adjusting water flow:

Water flow will be checked at the beginning of the study and adjusted to proposed pre-study flow rates (see Table 5.9.3). Water flow will be visually checked daily and adjusted if the Study Director, Investigator or their designee believes water flow has deviated substantially (>10%) from proposed pre-study flow rates. Water flow does not play a significant role in initiation of disease, and minor water flow fluctuations should have no bearing on test results.

5.10 Environmental conditions:

Trials will be conducted under environmental conditions which will be unique for each participating facility.

5.11 Tank Cleaning:

Tanks will be cleaned at the beginning of each day. However, some study sites do not have sufficient resources to clean raceways daily. If this is the case, raceways will be cleaned on a regular schedule, which will be described in the final report. Tank cleaning will entail brushing the bottom of test units. Standpipes will be loosened to draw re-suspended material down the drain. Drawn down water levels should not result in stress to fish by impingement against tail screen or by de-watering. Tank cleaning will be complete when standpipes are refitted into rearing unit drains.

5.12 Provisions for necropsy and disposal of expired test subjects:

In most cases, necropsies will not be preformed. Cause of death will be assumed to be by the pathogen identified prior to the study start. If a necropsy is to be preformed, necropsy procedures used will be those outlined in Procedures for the Detection and Identification of Certain Fish Pathogens, 3rd edition (Amos 1995; see Appendix VIII). For disposal of expired test subjects see Section 5.7.3.

5.13 Fate of living test animals after study completion:

The fate of all remaining test animals from studies conducted at or coordinated by the NIO will be left to the discretion of the Hatchery Manager or Biologist in Charge. However, if post-study fish are to be used as part of the facilities production program, they will be subjected to the withdrawal period prior to release. Control fish which still may be diseased may be treated according to the Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug (INAD) Exemption, Oxytetracycline - Feed Additive INAD #9006. In most cases, fish will likely be transferred back to the original production lot from which they came. If post-study fish are not to be part of a facilities production

program, they will be disposed of in a local landfill or incinerated. At this time, it is not possible to forecast the exact fate of living test animals after study completion at a particular facility. The fate of living test animals after study completion at a particular facility will be described in the final report.

5.14 Test unit configuration:

Test units may be different for each participating study site, depending upon a number of factors including species, fish size and tank availability. Test unit dimensions, as well as standpipe height, test unit volume (cubic feet and gallons) will be recorded on Form 3. Prior to transfer of fish into test units, standpipes will be put into place, water turned on and water flow rate adjusted to achieve desired flow index. Once a test unit is full, depth measurements will be taken at several locations to get an average depth. In circular test units, depth measurements will be taken at the outside edge of the tank, next to the standpipe, and midway between the outside edge and standpipe. In rectangular test units, depth measurements will be taken at the head, tail and midpoint of the tank. All measurements will be made near the mid-line of the test unit.

5.15 Owner consent:

Not applicable.

6 SPECIFICATIONS OF VARIABLES:

6.1 Primary variable to be measured for evaluating labeled claim:

Mortality will be the primary response variable. This is consistent with addressing the "proposed" label claim, which may read "to control or prevent mortality associated with Flavobacteriosis in a variety of Fish Species.

6.1.1 When primary variables will be assessed:

Mortalities will consist of dead fish. Mortalities will be removed, counted and recorded daily, beginning the first day of the treatment period and ending at 14 - 30 days post treatment. Mortalities will be removed and counted from each test unit at the beginning and end of each day.

6.1.2 Procedures for assessing primary variable:

Fish will be evaluated as either 'dead' or 'alive' by the Study Director, Investigator and/or hatchery biologist by observation. The study participant responsible for assessing which fish are dead will have had extensive experience in evaluating whether fish are dead or not based on

experience working at a fish hatchery (see Section 10 for list of Study Personnel and Appendix XIV for CV's).

6.1.3 Equipment used for assessing primary variable:
No specialized equipment will be required to evaluate or remove dead test animals.

6.1.4 Calculation of derived data:
Data used for statistical analysis will be total mortality during the entire study period. Total mortality will be the sum of the daily mortality during this period. If provisions are made to remove untreated test units from the study once mortality has been shown to be significantly different (higher) than among treated units, then data will be handled according to recommendations provided by CVM's Biometrics Division.

6.1.5 Forms for retention of source data:
Form 4 or similar form will be used to record mortality data.

6.1.6 Name(s) and address(es) of outside labs used for analysis:
No outside labs will be used for analysis of the primary response variable (mortality).

6.2 Other variables to be recorded during the study:
No other variables will be recorded for data analyses purposes.

6.2.1 When other variables will be assessed:
n/a

6.2.2 Procedures for assessing other variables:
n/a

6.2.3 Equipment used to assess other variables:
n/a

6.2.4 Calculation of derived data:
n/a

6.3 Adverse reactions:
Adverse reactions will be recorded and reported immediately to study monitor. Adverse reactions will be recorded on Form 9 or log book.

6.4 Study facilities:

Study facilities refer to the placement and location of test units at a particular study site. At a particular study site, all test units will be under the same conditions within the hatchery building.

6.4.1 Containment equipment:
Test fish will be contained in either fiberglass, aluminum or cement tanks, troughs or raceways. Each facility may use a different test unit, depending upon species requirements and tank availability. However, all test units used for a particular study will be the same. Type of test unit, dimensions and test unit volume will be recorded on Form 3

6.4.2 Lighting equipment:
No specialized lighting equipment will be used. Photoperiod will be identical among all test units at a particular facility. Photoperiod may, however, be different for different facilities, and will be described in the final report.

6.4.3 Heating equipment:
No specialized heating equipment will be used.

6.4.4 Cooling equipment:
No specialized cooling equipment will be used.

6.4.5 Feeding equipment:
No specialized feeding equipment will be used during the treatment period

6.4.6 Watering equipment:
No specialized watering equipment will be used.

6.4.7 Ventilation equipment:
No specialized ventilation equipment will be used:

6.4.8 Space allocation of test units:
All test units will be in the same area to ensure minimizing variability in conditions, which may affect the test unit or test animals within.

6.4.9 Pasture allocation:
Not applicable

6.4.10 Facility diagram:
If available, hatchery diagrams and tank configuration will be included in the final report

6.5 Experimental diets:
All diets used will be commercial diets normally used at the site where the study is to be conducted.

6.5.1 Drug concentration of commercial diet:
An HPLC method, developed by the staff at the USGS, UMESC, will be used to assay the feed to determine drug concentration

6.5.1.1 Assayed drug concentrations:
Oxytetracycline will usually be a 2, 4 or 6% premix, unless top-coated at the pivotal study site.

6.5.1.1.1 Drugs to be assayed in each treatment group:
Oxytetracycline

6.5.1.1.2 Anticipated analytical variation and assay limits:
This information is contained within files at the USGS UMESC and is available upon request.

6.5.1.1.3 Analytical method:
The title of the procedure to be used is Determination of Oxytetracycline in Fish Feed. See Section 8.0 Analytical Methods for a description of the method and Appendices where it can be found.

6.5.1.1.4 Analytical Laboratory:

6.5.1.1.4.1 Address:
Dr. Guy Stehly
Upper Midwest Environmental Science Center
2630 Fanta Reed Road, Box 818
La Crosse, Wisconsin 54602-0818

6.5.1.1.4.2 Telephone Number:
608-783-6451

6.5.1.1.5 Number of assay replicates:
Three samples will be taken from each lot of medicated feed used during the study. Samples should be collected at the beginning, middle and end of the study.

7 DATA ANALYSIS:

7.1 Define the experimental unit:

The experimental unit will be the test unit, not the individual fish.

7.2 Define the number of replicates per treatment:

There will be a minimum of three replicates per treatment, and there will be a minimum of two treatment conditions (treated and control). There will be at least a total of six test units used per study. In the event one test unit is excluded from the study (see exclusion criteria), statisticians from FDA may be consulted to determine the most appropriate statistical test to use.

7.3 Define statistical methodology:

7.3.1 Null hypothesis:

H_o : $u_1 = u_2$; Mortality caused by flavobacteriosis is equal between fish treated with 2.5 - 10.0 grams oxytetracycline medicated feed fed daily for 10 - 14 days and fish that receive no oxytetracycline medicated feed.

7.3.2 Alternate (research) hypothesis:

H_a : $u_1 \leq u_2$; Mortality caused by flavobacteriosis will be lower among fish treated with 2.5 - 10.0 grams oxytetracycline medicated feed fed daily for 10 - 14 days and fish that receive no oxytetracycline medicated feed.

7.3.3 Assumptions:

1) Two normally distributed populations.
2) Equality of variances is known.
3) Independent random samples of size n_1 and n_2.

7.3.4 Biostatistical procedures used:

An independent t-test will be used to detect differences between treated and untreated fish with regard to total fish mortality/test unit . Where differences are stated to be significant, a level of $p \leq 0.05$ is implied. Bartletts test for homogeneity of variance will be used to test for equality of variance. Where variances are equal, results from the pooled variances t-test will be used. Where variances are unequal, results from the separate variances t-test will be used. The separate variances t-test adjusts the degrees of freedom to account for unequal variances (see appendix VIII, page 601). If there is a potential for pooling data from different studies involving different fish species, discussions with CVM's Biometrics Division will be initiated to explore other statistical analyses.

7.3.5 Statistical data software to be used:

The statistical software package to be used will be SYSTAT (Wilkinson 1990) or equivalent.

7.4 Define how the statistical results will be used to draw conclusions about the study's objective:

Differences in mortality will be detected using an independent t-test. Where differences are stated to be significant, a level of $p \leq 0.05$ is implied. If total mortality among non-treated control groups is higher than total mortality among the treated groups, and the calculated *p* value is less than 0.05, then the conclusions drawn will state that the oxytetracycline medicated feed treatment regime used was effective in controlling mortality caused by flavobacteriosis in the test fish species used. If total mortality among non-treated groups is higher than the total mortality among treated groups, but the calculated *p* value is greater or equal to 0.05, or if the total mortality among non-treated groups is less than or equal to the total mortality among treated groups, then the conclusions drawn will state that the oxytetracycline medicated feed treatment regime used was not effective in controlling mortality caused by flavobacteriosis in the test fish.

8 ANALYTICAL METHODS:

8.1 Describe the analytical measurement to be made and the relevance to the protocol objective:

The only analytical measurement to be made is to confirm dose of oxytetracycline medicated feed used during the treatment period. This method is described in detail in the UMESC's Technical Operating Procedure for Determination of Oxytetracycline in Fish Feed (SOP No. CAP 416.0). This method involves extraction of analyte from feed using a McIlvane EDTA extraction buffer and analyzing samples using HPLC (high pressure liquid chromatography). Analytical confirmation of treatment dose is important to verify the working treatment concentration.

8.2 Specify the analytical plan to be used for the protocol measurements:

8.2.1 An abstract of the method:

This information is contained within files at the UMESC and is available upon request.

8.2.2 Description of procedures for sample selection, preparation, and storage:

This information is contained within files at the UMESC and is available upon request.

8.2.3 Evidence of methods validation:

This information is contained within files at the UMESC and is available upon request.

8.2.4 Description of validation method plan when method is being developed for the study:

This information is contained within files at the UMESC and is available upon request.

8.2.5 Quality control procedures for the method and criteria used to assess analytical results:

This information is contained within files at the UMESC and is available upon request.

8.3 Relevant scientific literature supporting the use of the analytical method for the intended measurements:

This information is contained within files at the UMESC and is available upon request.

8.4 Certification that all needed validations will be done before the initiation of the study:

This information is contained within files at the UMESC and is available upon request.

9 STUDY LOCATIONS:

Table 9. List of potential study location, address, phone number, and contact person (Hatchery Manager/Asst. Manager). If studies are done at facilities not listed below, location will be described in the final report.

Facility	Address	Phone Number	Contact
Coleman NFH	24411 Coleman Fish Hatchery Rd, Anderson California, 96007	916-365-8622	Roger Shudes Tom Nelson
Makah NFH	P.O. Box 739, Neah Bay, WA 98357	360-645-2521	Al Jensen
Quilcene NFH	281 Fish Hatchery Road, Quilcene, WA 98376	360-765-3334	Larry Telles
Natchitoches NFH	615 Highway 1, S Natchitoches, LA 71457	318-352-5324	Jan Dean Karen Kilpatrick

10 PERSONNEL:

Personnel will be debarred from participating in the proposed field based clinical efficacy trials if they take a new position within the Service, or take a new position outside the Service. It is not anticipated that justification for debarment will occur in the near future.

10.1 Investigators, study monitor and fish health biologists involved in proposed field based clinical efficacy trials:

See table 10.1 below. See appendix XIV for Curriculum Vitaes for Investigators, Study Monitor and Fish Health Biologists involved in the proposed field based clinical efficacy trials. If studies are conducted at facilities not listed in the table above, personnel involved in the study will be included in the final report.

Table 10.1 Investigators, study monitor and fish health biologists involved in proposed field based clinical efficacy trials.

Facility	Investigators and Phone Number	Study Monitor/Fish Health Biologist and Phone Number
Coleman NFH	John Scott; 916-365-8622	Scott Foott; 916-365-4271
Makah NFH	Al Jensen; 206-645-2521	Joy Evered; 360-753-9046
Quilcene NFH	Larry Telles; 360-765-3334	Joy Evered; 360-753-9046
Natchitoches NFH	Jan Dean; 318-352-5324	Norm Heil; 706-655-3382

10.2 Other personnel involved in study:

Other personnel involved in studies, and their CV's, will be listed and filed in the final report.

11. COLLECTION AND RETENTION OF SOURCE DATA:

All source data, including those produced electronically, and a copy of all applicable reports will be retained at the Service's National INAD Office (NIO) in Bozeman, MT in a secure area which protects the source data and records from deterioration, destruction, tampering and vandalism in accordance to Section D-4 paragraphs (a) and (b) of the Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (dated Oct. 1992 or later - see Attachment II). Also, copies of all source data will be stored in a similar manner at the original study site.

12 ADDENDUM/DEVIATIONS TO THE PROTOCOL:

12.1 Protocol addendums:

Protocol addendums will be forwarded to FDA/CVM and referenced to the Efficacy of Oxytetracycline Medicated Feed for Control of Mortality Associated with Flavobacteriosis a variety of Fish Species, Protocol OTF - 98 - EFF. Cover letters accompanying submitted addendums will reference the submittal date of the above protocol. Addendums will also be attached to this protocol (see Appendix XV).

12.2 Protocol amendments:

A signed copy of the Study Protocol must be retained by each Investigator. At any time before a study begins, desired changes in the Study Protocol should be brought to the attention of the Study Monitors. The desired changes will be fully described in the form of an amendment along with the reason for the change. The amendment will be signed by Dr. Erdahl. Copies of the signed amendment will be attached to each copy of the Study Protocol. Amendments will also be attached to this protocol (see Appendix XV).

12.3 Protocol deviations:

Deviations from the established Study Protocol occasionally cannot be avoided. If deviations occur, the Study Monitor will be contacted immediately for advice. Protocol deviations will be documented fully and accompanied by a written explanation of what happened, why, and what steps were taken to mitigate the deviation. Deviation statements will be signed and dated. These statements will be forwarded to the Study Monitor and then submitted with the protocol as part of the final report. Deviations will also be attached to this protocol (see Appendix XV).

13 DRUG DISPOSITION/ANIMAL ACCOUNTABILITY/FEED DISPOSITION/FEED ACCOUNTABILITY:

Unused drug remaining at the end of a study should be disposed of in a landfill or by burial. If by chance there is a bona fide need for unused drug-treated feed immediately following completion of a treatment regime, Investigators should consult with Study Monitors to determine if unused feed is appropriate for further use. Supplemental use of unused drug-treated feed is allow only with Study Monitor approval. The investigational drug may not be redistributed to others not specified by the Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Oxytetracycline - Feed Additive INAD #9006 (see Attachment I) and may not be retained by the Investigator after completion of the study. Fate of unused living test animals will be handled in a manner identical to the fate of living post-study test animals (see Section 5.12 - Fate of living test animals after study completion). In most cases, unused living fish will be returned to the general hatchery population.

REFERENCES

Amos, Kevin H. , editor. 1985. Procedures for the Detection and Identification of Certain Fish Pathogens. Third Edition. Fish Health Section, American Fisheries Society. Corvallis, Oregon.

Meyers, Theodore R., editor. 1997. Fish Pathology Section Laboratory Manual. Special Publication No. 12. Alaska Department of Fish and Game, Commercial Fisheries Management and Development. Juneau, Alaska

Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McCraren, L.G. Fowler and J.R. Leonard. 1982. Fish Hatchery Management. United States Department of the Interior, U.S. Fish and Wildlife Service, Washington, DC., 517 pp.

Spear, D.J., R.J.F. Markham, B. Despres, K. Whitman and N. MacNair. 1995. Examination of Gills from Salmonids with Bacterial Gill Disease using Monoclonal Antibody probes for *Flavobacterium branchiophilum* and *Cytophaga columnaris*. Journal of Veterinary Diagnostic Investigations. 7:500-505

United States Fish and Wildlife Service (USFWS). 1995. Introduction to Fish Health Management. United States Department of the Interior, Washington, DC 139 pp.

Wilkinson, L. 1990. SYSTAT: The System for Statistics. SYSTAT, Inc. Evanston, IL 677 pp.

FORMS

1. Shipment of drug - Section 2.1.11
2. Drug log - Section 2.1.11
3. Environmental and culture conditions prior to treatment initiation - Section 6.3.14.
4. Daily mortality - space for recording mortality twice daily and daily total - Section 6.1.5
5. Daily dissolved oxygen - Section 5.9.3.2
6. Daily water temperature - Section 5.9.3.2
7. pH and hardness - Section 5.9.3.2
8. Treatment Condition Code
9. Adverse reactions Section 6.4
10. Fish Health Evaluation
11. Disposal Record for Animals from Clinical Field Trials

APPENDICES:

I. Oxytetracycline MSDS

III. Standard fish health diagnostic scheme to confirm diagnosis of cold water disease, and determine if other infectious pathogens are present.

IV. Procedures for necropsy

V.

VI. Investigational Withdrawal Period

VII. Reference material for the following: 1) YSI - 55 DO meter, 2) procedure for determining pH, and 3) procedure for determining hardness (as $CaCO_3$).

VIII. Reference for appropriateness of using pooled and separate variances when using an independent t-test to detect differences between treated and untreated fish with regards to total fish mortality/test unit.

IX.

X.

XI.

XII.

XIII.

XIV. Curriculum vitaes for Study Monitors and Investigators

XV. Addendums/deviations to protocol.

FORM 1. GUIDE FOR REPORTING INVESTIGATIONAL NEW ANIMAL DRUG SHIPMENTS FOR POIKILOTHERMIC FOOD ANIMALS

Department of Health and Human Services
Center for Veterinary Medicine, HFV-199
Food and Drug Administration
7500 Standish Place
Rockville, Maryland 20855

Date:
I-1NAD No: 9006-1
Name of Drug: Oxytetracycline
Trial Number:
Lot Number:

The sponsor, U.S. Fish and Wildlife Service, submits a notice of claimed investigational exemption for the shipment or delivery of a new animal drug under the provisions of Section 512 of the Federal Food, Drug, and Cosmetic Act. The following information is submitted in triplicate (original and two copies):

Name of Drug: Oxytetracycline Feed Additive

Proposed Use of Drug: Treatment of cold water disease and columnaris (flexibacteriosis) in a variety of cultured fish species

Date of CVM Authorization Letter: **xxxxxx**

Date of Drug Order: _____

Amount of Drug Ordered: _____

Name of Investigator: _____
(typed or printed)

Location of Trial: _____

Pivotal (intended for support of NADA) X or nonpivotal _____ study

Approximate Number of Animals: Treated _____ Controls _____

Protocol (*pivotal studies only*): Date submitted to CVM and/or number: **xxxxxxxxx**

Approximate Date of Trial: Start: _____ End: _____

Species, Size, and Type of Animals: _____

Maximum dose and duration: 10.0 g active drug/100 lbs fish/day for up to 14 days

Method(s) of Administration: oral administration

Withdrawl Period: 21 - 40 days depending on treatment regime used

If the investigation is discontinued, the Food and Drug Administration will be notified, giving the reason and disposition of the drug.

Investigator: _____
Signature and Date

Study Monitor: _____
Signature and Date

Form 2. Chemical Use Log for Clinical Field Trials Using Oxytetracycline as a Feed Additive to Control Mortality caused by Flexibacteriosis.

Instructions:
1. Initiate Form 2 immediately upon receipt of Oxytetracycline Medicated Feed.
2. Only a single shipment/lot number of Oxytetracycline should be used per each copy of Form 2.
3. A signed copy of Form 2 should be sent to the NIO at the end of the Study Year.
4. Original Form 2 should be archived at the investigating facility.

Facility: _____ **Trial Number:** _____
Lot Number of Oxytetracycline: _____ **Date Received:** _____

Date Received	Amount Received (lbs)	Dates Used	Amount Used (lbs)	Species Treated or OTC-Feed Disposal	OTC-Feed on hand	Inventoried by (initials)

Investigator: _____
Signature and Date

Study Monitor: _____
Signature and Date

Form 3. **Test Site, Species Tested, Environmental and Culture Conditions, and Water Quality Parameters**

Test Site: _____

_____

_____

Oxytetracycline Lot Number _____ Test Site Elevation: _____

Fish Species (inculding scientific name): _____

Fish Source (originating facility/body of water): _____

Fish Lot Number: _____ Fish Length (in): _____ Fish Weight (g): _____

Test Unit Type (e.g. fiberglass/circular, aluminum/trough, concrete/raceway): _____

_____

Test Unit Dimensions (ft) : _____ Standpipe Heigth[1] (in): _____

Test Unit Volume[2] (cu. ft): _____ Test Unit Volume[2] (gal): _____

Number of Test Units:

Number of Control Test Units: _____ Number of Treated Test Units: _____

Number of Fish/Test Unit: _____ Total Number of Fish in Study: _____

Flow Index[3]: _____ Density Index[3]: _____

Water Temperature (°C): _____ Water Flow per Test Unit (gpm): _____

Dissolved Oxygen (mg/L): _____ Dissolved Oxygen (% saturation): _____

pH: _____ Water Hardness (mg/L $CaCO_3$): _____

Water Source (e.g. well, spring, creek, resorvoir, etc): _____

NOTE:
It is assumed that test conditions are identical for each test unit in the study. If not, please specify any irregularities on a separate sheet and attach to this form.

[1] Standpipe height refers to distance standpipe extends above bottom of test unit.
[2] See Section 5.14 for description of measuring test unit.
[3] See Section 5.1.2.1 and 5.1.2.2 for description of flow index and density index calculations. Assume first pass water.

Investigator: _____ **Study Monitor:** _____
Signature and Date **Signature and Date**

Form 4a. **Daily Mortality Record for Pivotal Studies Evaluating the Efficacy of Oxytetracycline**

Medicated Feed to Control Mortality caused by Flexibacteriosis

Daily Mortality

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials

NOTE: Mortality should be recorded twice daily, once early in the morning and again late in the afternoon (see Section 6.1). The sum of the two values should then be recorded as total daily mortality. Total Mortality for the entire study period should include the sum of all values for the 5-day treatment period and the 14-day post-treatment period

Study Monitor ________________________ Date __________
Signature

Investigator ________________________ Date __________
Signature

Form 4b. Daily Mortality Record for Pivotal Studies Evaluating the Efficacy of Oxytetracycline

Medicated Feed to Control Mortality caused by Flexibacteriosis

Daily Mortality

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials
Total Mortality							

Study Monitor _____ Date _____
____ Signature

Investigator _____ Date _____
____ Signature

Form 5. Daily Record for Dissolved Oxygen Levels in Pivotal Studies Evaluating the Efficacy of Oxytetracycline Medicated Feed to Control Mortality caused by Flexibacteriosis

Dissolved Oxygen (mg/L)

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Form 6. Daily Record for Water Temperature in Pivotal Studies Evaluating the Efficacy of

Oxytetracycline Medicated Feed to Control Mortality caused by Flexibacteriosis

Water Temperature (°C)

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Form 7. **Record of pH and Water Hardness in Pivotal Studies Evaluating the Efficacy of Oxytetracycline to Control Mortality caused by Flexibacteriosis**

pH and Water Hardness

Date	pH	mL titrant used to get color change	Water Hardness (mg/L $CaCO_3$)	Initials

NOTE: pH and water hardness at the beginning and end of the trial

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Raw Data: Water Hardness - Record number mLs titrant used to change sample color, and show calculations used to derive mg/L hardness (as CaCO3).

Form 8. Treatment Condition Code for Pivotal Studies Evaluating the Efficacy of Oxytetracycline Medicated Feed to Control Mortality caused by Flexibacteriosis

Treatment Condition Code

Treatment #1 Tank #1: _____	Control Tank #1: _____
Treatment #1 Tank #2: _____	Control Tank #2: _____
Treatment #1 Tank #3: _____	Control Tank #3: _____

NOTE: Once this form has been completed, it must be stored in a secure location not accessible to anyone except the listed hatchery manager/assistant manager. At study termination, this form is to be included with other data/information forms.

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Note: sign form at the end of the study

Form 9. Record of Observations/Adverse Reactions and/or Oxytetracycline Medicated Feed Toxicity

It is imperative that a complete record of possible adverse reactions and/or drug toxicity is established with respect to fumagillin treatment. This should include a description of all pertinent events before, during, and after treatment. The following space is provided for such information. If more space is required, please attach supplemental sheets to this form.

Date	Tank ID	Initials	Observations

Date	Tank ID	Initials	Observations

Date	Tank ID	Initials	Observations

Investigator: _____ _____
Signature Date

Study Monitor: _____ _____
Signature Date

Form 10. Fish Health Evaluation

Tank No. _____
Fish No. _____
Date _____

Fish species _____ Wt. (g) _____ Length (inches) _____

Body Surface: ( ) Normal ( ) Excess Mucus ( ) Irregular color
( ) Gross Pathology _____
( ) Micro Wet _____

Fins: ( ) Normal ( ) Gross Pathology: Frayed P1 P2 Ad C D An
Hemorrhaged P1 P2 Ad C D An
Eroded P1 P2 Ad C D An
( ) Micro Wet _____

Gills: ( ) Normal ( ) Pale ( ) Hemorrhagic ( ) Necrotic
( ) Micro Wet: ( ) Mucus _____ ( ) Hypertrophy F L _____
( ) Edema _____ ( ) Hyperplasia F L _____
( ) Embolism _____ ( ) Telangiectasis _____
( ) Bacteria _____
( ) Parasites _____

Liver: ( ) Normal ( ) Pale ( ) Edematous ( ) Mottled ( ) Fatty
( ) Hemorrhagic ( ) Necrotic
( ) Parasites _____

Spleen: ( ) Normal ( ) Pale ( ) Enlarged ( ) Edematous ( ) Granulated
( ) Parasites _____

Posterior Kidney: ( ) Normal ( ) Pale ( ) Edematous ( ) Necrotic
( ) Parasites _____

Dermal Lesion: ( ) None ( ) Ulceration ( ) Hemorrhagic ( ) Necrotic
( ) Furuncle ( ) Marginal ( ) Marginal
( ) Central ( ) Central
( ) Closed ( ) Open
( ) Location: ( ) Dorsal ( ) Caudal ( ) Lateral
( ) Ventral ( ) Cranial ( ) Isthmus
( ) Base of fins P1 P2 Ad C D An
( ) Description: _____
( ) Micro Wet _____

Notes and Comments on other organs and tissues

Eyes _____ Stomach _____
Body Cavity _____ Gastrointestinal Tract _____
Gall Bladder _____ Gas Bladder _____
Adipose Tissue _____ Musculature _____
Gonads _____ Brain _____
Other _____

Study Monitor _____ Date _____

Investigator _____ Date _____

Form 11. Disposal Record for Animals from Clinical Field Trials Using Oxytetracycline Medicated Feed to Control Mortality caused by Flexibacteriosis

Facility name and trial number: _____

Oxytetracycline Lot No.: _____ **Date Received:** _____

Fish Lot No.: _____ **Date of Disposal:** _____

Number of Fish: _____ **Size of Fish:** _____

Date of Last Treatment: _____

Disposal Method: ☐ Stocking ☐ Burial ☐ Incinerator

_____

_____

Disposal Site:

Location of Stocking Water: _____

Location of Burial Site: _____

Location of Incinerator: _____

Investigator: _____ _____
Signature **Date**

Study Monitor: _____ _____
Signature **Date**